ANALYSIS OF THE LABIAL GLAND SECRETION OF THE CUCKOO-BUMBLEBEE (*Psithyrus vestalis*) MALES AND SYNTHESIS OF ABUNDANT GERANYLCITRONELLOL

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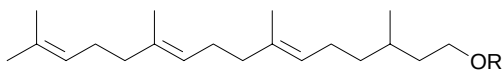
Dedicated to the memory of Dr Zdenek Arnold.

Labial glands of the cuckoo-bumblebee males of the species *Psithyrus vestalis* were extracted and the components of their secretions were identified. Chemical composition of the males' signal of *Psithyrus vestalis* has not yet been described in the literature. We found geranycitronellyl acetate to be the main component (48%). Geranylcitronellol and (*Z*)-15-eicosen-1-ol were present in the secretion in lower amounts. Long-chain aldehydes, acetates, and hydrocarbons formed only minor components of the mixture. The identification of minor components was based on GC-MS, while the more abundant compounds were isolated and fully characterized by spectral and chemical methods. For the structure confirmation, geranylcitronellol was prepared from geranyl bromide via a three-step synthesis.
Key words: *Psithyrus vestalis*; Cuckoo-bumblebees; Marking pheromone; Geranylcitronellol; Geranylcitronellyl acetate.

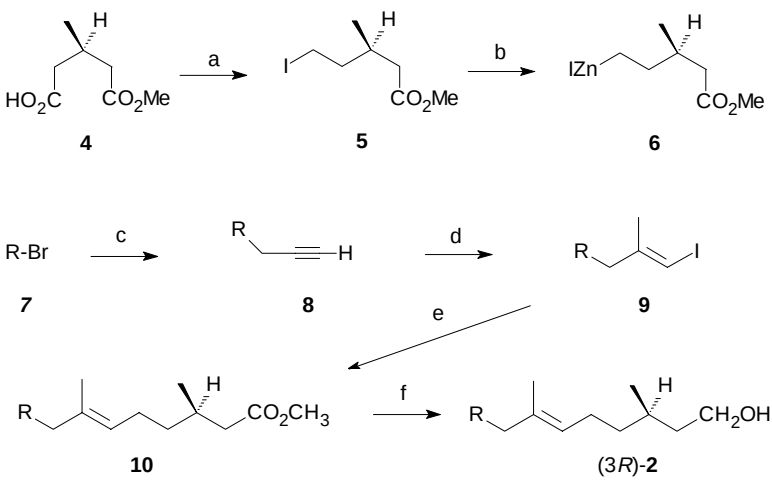
Chemical signals of bumblebees were studied extensively by Swedish authors[1,2]. During the flight period in summer, the bumblebee and cuckoo-bumblebee males in the majority of species exhibit a patrolling behaviour. They fly around the nest or away from it and mark their territories with a pheromone secreted from the labial gland[3,4]. The pheromone-marked places attract conspecific females for mating[5].

The labial gland secretion is usually a mixture of acyclic mono-, sesqui- and diterpenes (alcohols and acetates), various straight-chain fatty acid derivatives (alcohols, esters, aldehydes, and both saturated and unsaturated hydrocarbons with from 12 to 18 carbon atoms in the chain). The composition of the secretion is species-specific[1]. Bergström and co-workers studied the temporal and spatial segregation between species and subspecies[6]. Except for the geographical isolation, species patrolling in the same area segregate to some extent in time and space. Species occurring in the same time and habitat differ substantially in the composition of their marking pheromone to avoid inter-species mating.

Chemistry of several Scandinavian *Psithyrus* species was published by Cederberg and co-workers[7]. The occurrence of *Psithyrus vestalis* was earlier reported as very rare in Scandinavia, but its spreading in South-Western Sweden during last few years was found by Andersson[8]. The cuckoo-bumblebee species living in the Czech Republic have not been thoroughly investigated from the chemical point of view. The secretion of *Psithyrus vestalis* has not yet been described in the literature. Preliminary results on the labial gland secretion of *Psithyrus vestalis* were presented only recently[9]. However, the chemoecological aspects of this species will be published independently by Bergman and coworkers[10].



1, R = $COCH_3$ **2**, R = H



SCHEME 1

EXPERIMENTAL

Insects: Males of the species *Psithyrus vestalis* (9 individuals) were collected in August 1995 in Czech Central Mountains, locality Oblik, about 60 km North-West of Prague, elevation 509 m above the sea level. The collected living insects were transported to the laboratory and then kept in the freezer until dissection of labial glands. The dissected glands were extracted with hexane (50 μl per gland).

Chromatography: The extracts and synthetic samples were analyzed using a gas chromatograph HP 5890 (Hewlett–Packard) equipped with a split/splitless injector (220 °C), and with a flame ionization detector (290 °C) or a mass detector (Fisons MD 800) working in impact ionization mode, respectively. A DB-5 column (30 m × 0.25 mm, J & W Scientific) and helium gas (flow 0.7 ml/min at 50 °C) was used for the separations. The temperature program started at 50 °C (1 min delay), than temperature of the oven was increased to 140 °C (rate 50 °C/min), to 240 °C (2 °C/min), and finally to 320 °C (5 °C/min). The identification of minor compounds was based mostly on their mass spectra compared with those in National Institute of Standards and Technology (NIST, U.S.A.) Library and on the co-chromatography with synthetic or commercially available standards. Column chromatography separations were made on Merck 60 silica gel (0.040–0.063 mm) using ethyl acetate or ether in light petroleum or hexane[11]. Silica gel/silver nitrate chromatography was performed on a TLC plate (Merck 60 GS 254, 35 × 75 mm) impregnated with $AgNO_3$ (10%). A repeated elution with hexane–ether mixture (7 : 3) was used for the separation.

Spectral methods: NMR spectra were determined in $CDCl_3$ solutions on Varian UNITY-500, operating at 499.5 MHz for ^1H and at 128 MHz for ^{13}C NMR spectra, respectively. Chemical shifts are expressed in δ (ppm) scale relative to tetramethylsilane for ^1H and relative to $CDCl_3$ signal (77.23 ppm) for ^{13}C NMR, respectively. Coupling constants (*J*) are reported in Hz. Tentative assignments are denoted with an asterisk. IR spectra, reported in cm^{-1}, were recorded on a Bruker IFS 88 FT-IR and a Perkin–Elmer 621 spectrometers in CCl_4 solutions and in potassium bromide micropellets (1.5 mm diameter), respectively. Gas-phase infrared spectra (GC-FTIR) spectra were taken on a HP 5890 gas chromatograph coupled with an HP 5695A IRD equipped with a narrow-band (4 000–750 cm^{-1}) infrared detector (mercury cadmium telluride). Electron impact (70 eV) mass spectra were obtained on ZAB-EQ (VG, England) instrument. Optical rotation was determined on a Perkin–Elmer 241 polarimeter.

Chemicals: All chemical reactions were run in an oven dried glassware under inert atmosphere of argon or nitrogen. Allene was prepared from commercially available 2,3-dichloro-1-propene (Fluka) according literature procedure[12] and stored at –70 °C prior use. Tetrahydrofuran and ether were distilled from Na-benzophenone ketyl in nitrogen atmosphere. Extracts of reaction mixture were washed with aqueous sodium chloride solution, dried over $MgSO_4$ and solvents were removed at reduced pressure.

Preparative Chromatography of the Gland Extracts

The hexane extract of one gland (50 μl) was chromatographed on silica gel (290 mg) in a Pasteur pipette. The elution of sample started with pentane, followed by hexane–ether mixtures (1–40% of ether). Two fractions contained compounds concentrated enough to enable further separation and derivatization. The first one, eluted with 5% ether in hexane, contained geranylcitronellyl acetate **1**: Mass spectrum, *m/z* (%): 334 (0.7; M^+), 319 (0.2), 291 (1), 265 (2), 250 (1), 231 (0.2), 223 (1), 205 (1), 191 (2), 177 (1), 163 (4), 149 (5), 136 (17), 121 (21), 109 (8), 107 (8), 95 (28), 81 (78), 69 (100), 55 (12), 41 (35). IR spectrum (KBr): 1 742, 1 237, 1 042 (OAc). The second fraction, eluted with 40% ether in hexane, contained alcohols, where geranylcitronellol **2** and (*Z*)-15-eicosen-1-ol **3** prevailed. This mixture was separated on silica gel/silver nitrate preparative TLC. Geranylcitronellol **2** exhibited identical MS and IR spectral data as we are reporting for synthetic sample of (3*R*)-**2**. (*Z*)-15-

eicosen-1-ol: Mass spectrum, *m/z* (%): 281 (0.05; M^+–CH_3), 278 (0.1; M^+–H_2O), 250 (0.04), 152 (0.3), 138 (1), 137 (1), 124 (2), 123 (2), 110 (5), 109 (5), 96 (18), 82 (33), 81 (27), 69 (38), 67 (34), 55 (100), 41 (69), 31 (18). IR spectrum (KBr): 3 376 (OH), 2 957, 2 922, 2 852 (C–H), 1 460, 1 375 (C–H), 1 050 (C–O); (gas phase): 3 668 (OH), 3 012 (C=C), 2 994, 2 865, 1 460, 1 354, 1 045.

Derivatization: Dimethyl disulfide (DMDS) adduct of isolated alcohol **3** was prepared according published procedure[13]. Mass spectrum, *m/z* (%): 390 (12; M^+), 117 (100; $C_6H_{13}S$), 273 (99.6; $C_{16}H_{33}OS$). The same derivatization method was applied on a chromatographic fraction eluted with 2.5% ether in hexane and containing a mixture of hydrocarbons, aldehydes and acetates. The resulting mixture of aducts was then analyzed on GC-MS. The identified compounds are summarized in Table I.

Methyl (3*S*)-5-Iodo-3-methylpentanoate **5**

Methyl hydrogen (*R*)-(+)-3-methylglutarate **4** (Aldrich, 1 g, 6.3 mmol) was reduced $BH_3 . Me_2S$ (Aldrich, 0.8 ml, ca 8 mmol) using Rossi procedure[14]. Pentane solution of crude product was filtered over silica gel provided crude hydroxy ester (0.91 g) which was dissolved in dry pyridine (4 ml) and the solution was treated with 4-toluenesulfonyl chloride (1.20 g, 6.2 mmol) at 0 °C for 1 h and left in freezer overnight. The reaction mixture was quenched with ice, extracted with ether (3 × 15 ml) and combined extracts were washed with saturated aqueous $CuSO_4$ solution. Crude tosylate (0.81 g, 2.70 mmol) was stirred with NaI (0.61 g, 4.08 mmol) in dry acetone (15 ml) at room temperature for 20 h. Solvent was evaporated at reduced pressure, obtained oil was dissolved in pentane (20 ml) and washed with water (20 ml). Column silica gel chromatography yielded 368 mg (53% yield on starting **4**) of iodide **5**, $[\alpha]_D$ +1.87° (*c* 3.0, $CHCl_3$). ^{13}C NMR spectrum: 3.49 (C-5), 18.75 (C-6), 31.29 (C-3), 40.22 (C-4*), 40.61 (C-2*), 51.45 (CH_3), 172.88 (C-1). ^1H NMR spectrum: 0.97 d, 2 H, *J* = 6.6 (CH_3-3); 1.74 ddt, 1 H, *J* = 5.8, 8.0, 8.0, 14.0 (H-4); 1.92 dddd, 1 H, *J* = 5.4, 7.1, 8.6, 14.0 (H-4); 2.10 m, 1 H (H-3); 2.19 dd, 1 H, *J* = 7.8, 14.9 (H-2); 2.32 dd, 1 H, *J* = 6.1, 14.9 (H-2); 3.16 ddd, 1 H, *J* = 7.3, 8.3, 9.7 (H-5); 3.24 ddd, 1 H, *J* = 5.8, 8.5, 9.8 (H-5); 3.68 s, 3 H (CO_2CH_3). For $C_7H_{13}IO_2$ (256.1) calculated: 32.83% C, 5.12% H, 49.55% I; found: 32.77% C, 5.05 % H, 49.24% I.

(*E*)-6,10-Dimethyl-5,9-undecadien-1-yne **8**

Following the published procedure[15] a solution of allene (5 ml, 83 mmol) in dry ether (30 ml) was metallated with butyllithium solution in hexanes (2.4 M, 23 ml) at –70 °C for 1 h, warmed to –20 °C and stirred at this temperature for 0.5 h. The formed white precipitate of allene dianion was treated with geranyl bromide (**7**, 3.58 g, 16.5 mmol) at –35 to –20 °C for 18 min and the reaction mixture was allowed to warm to a room temperature and stirred for 30 min. The reaction mixture was poured over ice and extracted with pentane. Column chromatography on silica gel in pentane provided 1.421 g (49% yield on **7**) of **8**. ^{13}C NMR spectrum: 16.33 (CH_3-6), 17.91 (CH_3-10), 19.13 (C-3), 25.91 (C-11), 26.83 (C-8), 27.39 (C-4), 39.85 (C-7), 68.29 (C-1), 84.78 (C-2), 122.65 (C-5), 124.42 (C-9), 131.62 (C-10), 136.95 (C-6). ^1H NMR spectrum: 1.60 s, 3 H (CH_3-6); 1.62 s, 3 H (CH_3-10); 1.68 d, 3 H, *J* = 0.6 (3 × H-11); 1.94 t, 1 H, *J* = 2.4 (H-1); 2.00 t, 2 H, *J* = 7.3 (H-7); 2.09 dt, 2 H, *J* = 7.3, 7.3 (2 × H-8); 2.20–2.24 m, 4 H (2 × H-4, 2 × H-3); 5.10 tt, 1 H, *J* = 6.8, 1.2 (H-5); 5.18 bt, 1 H, *J* = 6.6 (H-9). For $C_{13}H_{20}$ (176.3) calculated: 88.58% C, 11.42% H; found: 87.82% C, 11.64% H.

(1*E*,5*E*)-1-Iodo-2,6,10-trimethyl-1,5,9-undecatriene[16] **9**

A solution of dicyclopentadienylzirconium dichloride (422 mg, 1.44 mmol) in dry dichloromethane (4 ml) was carefully treated with trimethylallane toluene solution (1 M, 1.44 ml) at room temperature followed by acetylene **8** (470 mg, 1.44 mmol) solution in dichloromethane (1 ml). The formed yellow

TABLE I
Compounds found in the labial gland of *Psithyrus vestalis* and their physical properties

Compound[a]	Retention time min	Relative proportions %	Mass spectral fragments *m/z*	Characteristic mass spectral fragments of DMDS adducts
Tetradecyl acetate	27.62	0.1	43, 55, 61, 69, 83, 168	
9-Hexadecenyl acetate	35.87	1.5	43, 55, 61, 67, 82, 222	145, 171, 231, 376
11-Hexadecenyl acetate	36.29	0.1	43, 55, 61, 67, 82, 222	117, 199, 259, 376
11-Octadecenal	36.42	0.1	41, 55, 67, 69, 81, 109	145, 215, 360
Octadecanal	37.42	0.1	41, 43, 55, 57, 67, 69, 82	
Geranylcitronellol **2**	43.34	8.2	41, 69, 81, 95, 163, 292	
Octadecadienyl acetate	44.59	1.1	43, 55, 61, 67, 81, 95	adduct not found
5,9-Heneicosadiene	45.54	14.5	55, 67, 81, 95, 235, 292	adduct *A*: 171, 215, 291, 339 adduct *B*: 117, 269, 291, 339
Heneicosadiene	45.64	4.1	55, 67, 81, 95, 250, 292	adduct not found
15-Eicosenal	46.39	6.0	55, 69, 83, 91, 276, 294	117, 271, 388
Geranylcitronellyl acetate **1**	48.34	47.8	69, 81, 95, 265, 291, 334	
(*Z*)-15-Eicosen-1-ol **3**	49.15	4.1	31, 55, 69, 82, 250, 278	117, 273, 390
Tricosane	50.17	3.6	55, 71, 85, 99, 113, 324	
Eicosadienyl acetate	53.42	3.5	43, 55, 61, 67, 81, 276, 336	adduct not found
15-Eicosenyl acetate	54.25	1.0	43, 55, 61, 67, 82, 278	117, 255, 315, 432
Pentacosane	58.39	1.7	55, 71, 85, 99, 113, 352	
Hexacosane	61.57	0.1	55, 71, 85, 99, 113, 366	
Heptacosane	64.25	1.7	55, 71, 85, 99, 113, 380	
Octacosane	68.64	0.5	55, 71, 85, 99, 113, 394	

[a] The compounds are listed in the elution order on a DB-5 column (5% phenyl methyl silicone); temperature program: 50 °C for 1 min; to 140 °C,

solution was stirred for 20 h at room temperature, cooled to 0 °C and a solution of iodine (440 mg, 1.73 mmol) in dry tetrahydrofuran (2 ml) was added. After 30 min the reaction was stopped by a careful adding of cold water and extracted with pentane–ether mixture (1 : 1). Purification on a silica gel afforded 125 mg of **9** (27%). ^{13}C NMR spectrum: 16.23 (CH_3-6*), 17.93 (CH_3-10*), 24.16 (CH_3-2), 25.94 (C-11), 26.48 (C-8*), 26.91 (C-4*), 39.72 (C-7*), 39.89 (C-3*), 74.94 (C-1), 123.17 (C-5), 124.25 (C-9), 131.63 (C-10), 136.26 (C-6), 148.04 (C-2). ^{1}H NMR spectrum: 1.59 s, 3 H (CH_3-10); 1.60 s, 3 H (3 × H-11); 1.69 s, 3 H (CH_3-6); 1.84 s, 3 H (CH_3-2); 1.99 dt, 2 H, *J* = 7.9, 6.7 (2 × H-8); 2.05 t, 2 H, *J* = 7.6 (2 × H-7); 2.12 dt, 2 H, *J* = 7.4, 6.7 (2 × H-4); 2.22 t, 2 H, *J* = 7.5 (2 × H-3); 5.13–5.03 m, 2 H (H-5, H-9); 5.87 s, 1 H (H-1). Mass spectrum, *m/z* (%): 249 (M^+ – 69, 1), 191 (M^+ – 127, 9), 182 (6), 149 (2), 135 (7), 121 (11), 109 (6), 107 (12), 81 (29), 69 (100), 67 (10), 55 (10), 54 (19), 41 (47). For $C_{14}H_{23}I$ (318.2) calculated: 52.83% C, 7.29% H, 39.87% I; found: 52.94% C, 7.80% H, and 39.95% I.

(3*R*)-Geranylcitronellol (3*R*)-**2**

a) *Formation of zinciodide* **6** (ref.[17]): Zinc dust (85 mg, 1.3 mmol) was suspended in dry tetrahydrofuran (0.2 ml) and subsequently activated with 1,2-dibromoethane (10 μl) and chlorotrimethylsilane (10 μl). A solution of iodide **5** (256 mg, 1 mmol) in tetrahydrofuran (0.7 ml) was added to the gray suspension of activated Zn at 25 °C during 20 min and the mixture was heated at 35–40 °C for 25 h. A clear solution of Zn organometallic compound **6** was obtained.

b) *Pd catalyzed cross-coupling*: Tetrakistriphenylphosphine palladium complex (44 mg, 0.04 mmol) was dissolved in a solution of iodide **9** (124 mg, 0.39 mmol) in dry tetrahydrofuran (0.5 ml) on yellow-brown solution. The solution of **6** (0.5 ml) prepared ad *a*) was added during 5 min, and the mixture was stirred for 48 h. Solvents were removed at reduced pressure, the obtained residue dissolved in hexane (2 ml) and poured on silica gel column. Elution with hexane–ether mixture (1 : 1) afforded methyl ester **10** (16 mg, 13% yield) which was reduced with $LiAlH_4$ (10 mg, 0.25 mmol) in ether (0.3 ml) forming (3*R*)-**2** (14 mg, 98% yield). $[\alpha]_D$ +5.2° (*c* 0.2, $CHCl_3$). ^{13}C NMR spectrum: 15.95, 19.54, 25.35, 25.65, 26.61, 26.76, 29.23, 29.40, 37.20, 39.71, 39.92, 62.24, 124.25, 124.43, 124.62, 131.27, 134.92, 134.94. ^{1}H NMR spectrum: 0.91 d, 3 H, *J* = 6.6 (CH_3-3); 1.04–1.72 m, 5 H (2 × H-2, H-3, 2 × H-4); 1.60 m, 3 H (CH_3-15*); 1.60 m, 6 H, (CH_3-11, CH_3-7); 1.68 q, *J* = 1.2 (3 × H-16); 1.95–2.09 m, 10 H, (2 × H-5, 2 × H-8, 2 × H-9, 2 × H-12, 2 × H-13); 3.68 m 2 H (2 × H-1); 5.08–5.13 m, 3 H (H-6, H-10, H-14). Mass spectrum, *m/z* (%): 292 (2; M^+), 277 (0.3), 249 (2), 223 (4), 208 (2), 191 (3), 179 (2), 163 (4), 149 (5), 136 (23), 121 (19), 109 (16), 95 (33), 81 (66), 69 (100), 55 (21), 41 (53). IR spectrum (KBr): 3 348 (OH), 2 963, 2 923, 2 872, 2 855 (C–H), 1 446, 1 375 (C–H), 1 060 (C–O). For $C_{20}H_{33}O$ (292.5) calculated: 82.12% C, 12.40% H; found: 82.60% C, 12.03% H.

RESULTS AND DISCUSSION

Nineteen compounds were found in the labial gland secretions of the *Psithyrus vestalis* males (Table I). The chemical structures of the compounds were determined based on spectral methods (MS and IR) and NIST spectral library. Some of the abundant components were isolated by micro-preparative column chromatography and TLC on silver nitrate impregnated silica gel and the chemical identity confirmed by derivatizations and spectral measurements. The alkalic hydrolysis of the isolated diterpenic acetate gave a product of the same retention time and the mass spectrum as those of synthetic sample of geranylcitronellol (3*R*-**2**). We concluded that this acetate is geranylcitronellyl acetate **1**. The position of the double bond in eicosen-1-ol was determined based on

mass spectra of the prepared DMDS adduct. The fragments 117 and 273 (*m/z*) showed that the double bond is located in the position 15. The configuration of the double bond is based on infrared spectra in gas phase[18]. The presence of the 3 012 cm^{-1} band (=C–H strech), and the absence of a 890 cm^{-1} band (*E*-wagg) in the measured gas phase IR spectrum of 15-eicosenol established the *Z*-configuration of the double bond.

The derivatization with DMDS is a very good method for the determination of the double bond position expecially in monounsaturated compounds. We were able to determine the double bond positions in all our monounsaturated components of the mixture, even the low abundant ones (Table I). However, the determination of the positions of double bonds in dienic compounds was not straightforward. Thus, the more abundant isomer of heneicosadiene (14.5%) gave two adducts, each showing the addition of one DMDS molecule. One product showed the substitution of the 5,6-double bond (*m/z* 117 and 269), while the second product had the two $S–CH_3$ substituents in the position 9,10 (*m/z* 171 and 215). Other DMDS adducts could not be found in the derivatized mixture (Table I). The configurations of double bonds of minor components of the extract could not be determined, however they have most likely *Z*-configuration. The positions of the double bonds in all our identified unsaturated products were in agreement with biosynthetic considerations published in the literature[19].

Synthetic sample of (3*R*)-**2** was prepared according to Scheme 1. Our synthetic procedure represents an efficient [3 + 1 + 6] extension procedure on geranyl bromide **7** which could be easily adopted to form both enantiomers of **2** and also other chiral acyclic terpenoids. Geranylcitronellol **2** was prepared previously by Ahlquist[20] using Kolbe coupling of acids and later by Janicke[21] starting from geranyl tosylate.

Geranylcitronellyl acetate **1** is the main component (47.8%) of the labial gland. This compound has not been found in bumblebees before, however, the corresponding alcohol **2** is rather common. Geranylcitronellol **2** is a medium-abundant component (8.2%) in our extract and has previously been found in other bumblebees and cuckoo-bumblebees, namely in *Bombus terrestris*[1], *Bombus hypnorum*[22], *Bombus lapponicus*[23], *Psithyrus rupestris*[7], and it also formed a minor component in other bumblebee species. (*Z*)-15-Eicosen-1-ol, one of the lower-abundant components (4.1%), was earlier found in *Psithyrus bohemicus*[19], a species the most closely related to *P. vestalis* in taxonomy (the two mentioned *Psithyrus* species form the subgenus *Ashtonipsithyrus*[24]).

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